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## **Amphibian immune defenses against chytridiomycosis: impacts of changing environments**

Rollins-Smith, Louise A ; Ramsey, J P ; Pask, J D ; Reinert, L K ; Woodhams, Douglas C

**Abstract:** Eco-immunology is the field of study that attempts to understand the functions of the immune system in the context of the host's environment. Amphibians are currently suffering devastating declines and extinctions in nearly all parts of the world due to the emerging infectious disease chytridiomycosis caused by the chytrid fungus, *Batrachochytrium dendrobatidis*. Because chytridiomycosis is a skin infection and remains confined to the skin, immune defenses of the skin are critical for survival. Skin defenses include secreted antimicrobial peptides and immunoglobulins as well as antifungal metabolites produced by symbiotic skin bacteria. Low temperatures, toxic chemicals, and stress inhibit the immune system and may impair natural defenses against *B. dendrobatidis*. Tadpoles' mouth parts can be infected by *B. dendrobatidis*. Damage to the mouth parts can impair growth, and the affected tadpoles maintain the pathogen in the environment even when adults have dispersed. Newly metamorphosing frogs appear to be especially vulnerable to infection and to the lethal effects of this pathogen because the immune system undergoes a dramatic reorganization at metamorphosis, and postmetamorphic defenses are not yet mature. Here we review our current understanding of amphibian immune defenses against *B. dendrobatidis* and the ability of the pathogen to resist those defenses. We also briefly review what is known about the impacts of temperature, environmental chemicals, and stress on the host-pathogen interactions and suggest future directions for research.

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## SYMPOSIUM

## Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments

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**Synopsis** Eco-immunology is the field of study that attempts to understand the functions of the immune system in the context of the host's environment. Amphibians are currently suffering devastating declines and extinctions in nearly all parts of the world due to the emerging infectious disease chytridiomycosis caused by the chytrid fungus, *Batrachochytrium dendrobatidis*. Because chytridiomycosis is a skin infection and remains confined to the skin, immune defenses of the skin are critical for survival. Skin defenses include secreted antimicrobial peptides and immunoglobulins as well as antifungal metabolites produced by symbiotic skin bacteria. Low temperatures, toxic chemicals, and stress inhibit the immune system and may impair natural defenses against *B. dendrobatidis*. Tadpoles' mouth parts can be infected by *B. dendrobatidis*. Damage to the mouth parts can impair growth, and the affected tadpoles maintain the pathogen in the environment even when adults have dispersed. Newly metamorphosing frogs appear to be especially vulnerable to infection and to the lethal effects of this pathogen because the immune system undergoes a dramatic reorganization at metamorphosis, and postmetamorphic defenses are not yet mature. Here we review our current understanding of amphibian immune defenses against *B. dendrobatidis* and the ability of the pathogen to resist those defenses. We also briefly review what is known about the impacts of temperature, environmental chemicals, and stress on the host–pathogen interactions and suggest future directions for research.

## Introduction

Amphibian species worldwide are experiencing the most severe population declines in recent history (Stuart et al. 2004; reviewed by Lannoo 2005; Skerratt et al. 2007; Collins and Crump 2009; IUCN Red List of Threatened Species 2011; Heatwole and Barrio-Amorós 2011; Heatwole et al. 2011). Although causes of these population declines are complex (Heatwole and Wilkinson 2009, 2011), a newly emerging fungal pathogen, *Batrachochytrium dendrobatidis*, results in development of the disease chytridiomycosis (Berger et al. 1998; Longcore et al.

1999; Pessier et al. 1999; reviewed by Berger et al. 2009) and is thought to be the leading cause of so-called “enigmatic declines” (Stuart et al. 2004). *Batrachochytrium dendrobatidis* is a unique fungal pathogen. It has a swimming zoospore as the infectious stage (Longcore et al. 1999; reviewed by Berger 2009) and is most closely related to other fungi that hold a basal position in fungal evolution (James et al. 2007). *Batrachochytrium dendrobatidis* colonizes skin cells of adult amphibians and the mouthparts of tadpoles (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999; Berger et al. 2005; reviewed by

Berger 2009). Infection occurs when zoospores land on the skin or the mouthparts and encyst (stop movement and settle, resorb the flagellum, and form a cell wall) (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999; Berger et al. 2005). Through mechanisms that are not understood, the pathogen moves from the surface of the skin to the *stratum granulosum* of the epidermis and matures in the *stratum corneum*. There, it enters healthy cells, grows in size, and develops into an urn-shaped zoosporangium in which the zoospores develop. As infected skin cells move toward the surface, the zoosporangium matures, the discharge papilla opens, and mature zoospores swim out (Berger et al. 1998, 2005; Longcore et al. 1999; Pessier et al. 1999). Zoospores are motile for about 24 h at 23°C before they encyst or die, but they survive more than 48 h at 4–14°C (Piotrowski et al. 2001; Woodhams et al. 2008). Movement is directed toward nutritional cues such as glucose, lactose, cysteine, and keratin (a major component of amphibian skin cells) (Moss et al. 2008). The mechanism by which this pathogen causes chytridiomycosis and death appears to be by interference with the ion transport functions of the skin, leading ultimately to cardiac arrest (Voyles et al. 2007, 2009). Unlike other fungal pathogens that move from the site of infection to other organs, *B. dendrobatidis* remains confined to the skin or tadpoles' mouth parts.

### Overview of Immune defenses against *B. dendrobatidis*

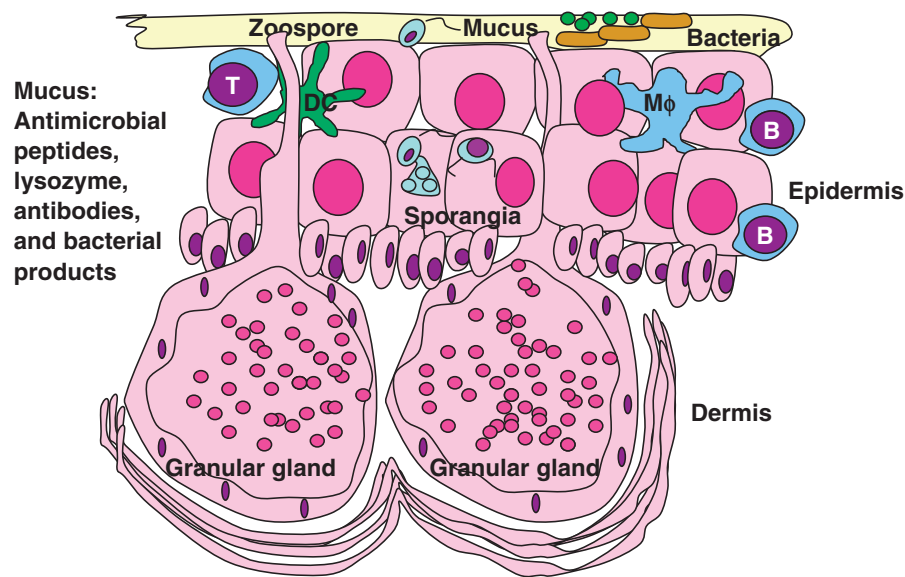
Because the zoospore must attach to the outer surface of the skin in order to establish infection, it must first overcome any defenses present in the skin mucus. The mucus contains secreted antimicrobial peptides (reviewed by Erspamer 1994; Rollins-Smith and Conlon 2005; Rollins-Smith 2009) and lysozymes (Zhao et al. 2006). We have recently shown that the mucus of the South African clawed frog, *Xenopus laevis*, also contains antibodies capable of binding to *B. dendrobatidis* (Ramsey et al. 2010). In addition to these natural defenses by the host, the mucus may also contain the metabolic products of symbiotic bacteria (Harris et al. 2006, 2009b; Brucker et al. 2008a, 2008b). When the pathogen enters cells of the *stratum granulosum* and *stratum corneum* of the epidermis, it would presumably alter the properties of the host's cells. Inflammation is reported in up to 40% of skin sites sampled in the highly susceptible species *Litoria caerulea* (Berger et al. 2005) and may consist of infiltration of neutrophils, lymphocytes,

and macrophages (Pessier et al. 1999; Nichols et al. 2001) (Fig. 1). We have recently shown that sublethal gamma irradiation reduces lymphocyte numbers and results in increased intensity of infection following experimental infection (Ramsey et al. 2010). Taken together, these results suggest that an adaptive lymphocyte-mediated immune response should develop to clear the infection. However, development of an effective cell-mediated defense appears to be impaired. Work that is currently in progress shows that *B. dendrobatidis* releases factors that inhibit lymphocyte proliferation in vitro by induction of apoptosis (J. Ramsey et al., unpublished data). Thus, the explanation for ineffective cell-mediated immunity may lie in the ability of the fungus to release immunosuppressive factors.

### Antimicrobial peptide defenses against *B. dendrobatidis*

The skin of many amphibians is rich in mucous and granular glands (also called poison or serous glands) in the dermis (Noble and Noble 1944; Bovbjerg 1963; Fox 1994). The mucous glands produce a material rich in heavily glycosylated mucins (Schumacher et al. 1994) and mucopolysaccharides (Duellman and Treub 1986) that help to keep the skin moist. Granular glands produce bioactive peptides including neuropeptides and antimicrobial peptides that are thought to play a role in defense against predators as well as microbes (reviewed by Barthalmus 1994; Erspamer 1994; Nicolas and Mor 1995; Zasloff 2002; Apponyi et al. 2004). Both mucous and granular glands are composed of a layer of epithelial cells surrounding a secretory compartment (Dockray and Hopkins 1975; Mills and Prum 1984). In granular glands, the epithelial cells fuse to form a syncytium, and the center of the gland is filled with granules packed with active peptides (Dockray and Hopkins 1975; Giovannini et al. 1987). Granular glands are surrounded by a layer of myoepithelial cells with sympathetic axons terminating between the cells (Sjoberg and Flock 1976). The myoepithelial cells possess  $\alpha$ -adrenoreceptors, and epinephrine or norepinephrine induce contraction and release of the contents (Benson and Hadley 1969; Dockray and Hopkins 1975; Holmes and Balls 1978). After discharge, the gland regenerates and peptide contents are restored (Neuwirth et al. 1979; Toledo and Jared 1995; Ramsey et al. 2010).

Amphibian antimicrobial peptides are active against Gram positive and Gram negative bacteria, fungi, protozoa, and viruses (reviewed by Nicolas



**Fig. 1** Schematic diagram of immune defenses against *Batrachochytrium dendrobatidis* in the skin. Mucus contains antimicrobial peptides, lysozyme, antibodies, and possible inhibitory bacterial products. The epidermis and dermis contain antigen-presenting cells such as lymphoid dendritic cells (DC), macrophages (Mφ), B-lymphocytes and T-lymphocytes.

and Mor 1995; Zasloff 2002; Apponyi et al. 2004). Although families of peptides are shared by related species, there is very little overlap in peptides from one species to another (reviewed by Conlon et al. 2004). Amphibian antimicrobial peptides are usually cationic, hydrophobic, and have the ability to form an amphipathic  $\alpha$ -helix in a membrane-mimetic environment (reviewed by Yeaman and Yount 2003). This structure bestows an ability to disturb biological membranes, and this seems to be an important mechanism of killing their targets. As more amphibian species are studied, we have learned that although many amphibian species synthesize and release cationic alpha-helical antimicrobial peptides, a number of other species appear to lack conventional antimicrobial peptides (Conlon 2011). Among anurans, antimicrobial peptides have been identified in species belonging to the families Alytidae, Bombinatoridae, Hylidae, Hyperoliidae, Leiopelmatidae, Leptodactylidae, Myobatrachidae, Pipidae, and Ranidae (Conlon 2011). Among other species examined, no cationic alpha-helical antimicrobial peptides have been found in members of the families Bufonidae, Ceratophryidae, Dicroglossidae, Eleutherodactylidae, Microhylidae, Pelobatidae, Pyxicephalidae, Rhacophoridae, and Scaphiopodidae (Conlon 2011). Thus, some species may have the capacity to secrete antimicrobial peptides, but antimicrobial peptides are only one of a number of immune defenses that amphibians employ in protecting their skin.

We and others have developed evidence that antimicrobial peptides play an important role in

protection from infection by skin pathogens such as *B. dendrobatidis* in the species that produce them. Individual purified antimicrobial peptides inhibit growth of *B. dendrobatidis* in vitro (reviewed by Rollins-Smith and Conlon 2005; Rollins-Smith 2009), and some inhibit growth of another fungus, *Basidiobolus ranarum* (Rollins-Smith et al. 2002a). Two of the peptides from *Xenopus laevis* act synergistically in inhibiting growth of both *B. dendrobatidis* and *Basidiobolus* (Rollins-Smith et al. 2002a), and natural mixtures of peptides from skin secretions are highly effective in inhibiting the growth of *B. dendrobatidis* (reviewed by Rollins-Smith 2009). The peptides are more effective against the zoospores than against mature sporangia (Rollins-Smith et al. 2002b, 2002c). Amphibian antimicrobial peptides also inhibit the infectivity of ranaviruses, another category of emerging pathogens in amphibians (Chinchar et al. 2001, 2004). We have shown that resting *X. laevis* and northern leopard frogs, *Rana pipiens*, constitutively produce and secrete small amounts of antimicrobial peptides onto the skin. The amounts of defensive peptides are dramatically increased when the frogs are chased, simulating a predator attack, and the concentrations are sufficient to inhibit chytrid growth (Ramsey et al. 2010; Pask et al., submitted for publication). Thus, the concentrations present on the skin of some normally active amphibians appear to be sufficient to inhibit colonization by *B. dendrobatidis*. Furthermore, depletion of antimicrobial peptides in *X. laevis* results in greater susceptibility to *B. dendrobatidis* infection (Ramsey



et al. 2010). This evidence supports the general hypothesis that antimicrobial peptides are an important component of the innate defense against skin pathogens. In addition to these laboratory studies, there is increasing evidence that the suite of antimicrobial peptides expressed by each species may predict whether they survive or decline when confronted with natural *B. dendrobatidis* infections in the wild. Species expressing a mixture of skin peptides that potentially inhibits *B. dendrobatidis* growth in vitro are among the more common and resistant species. In contrast, declining or endangered species express peptides with poor in vitro anti-*B. dendrobatidis* activity (Woodhams et al. 2006a). Among Panamanian species that have experienced declines, a few species that express skin peptides with strong anti-*B. dendrobatidis* activity are able to persist in geographic areas that have experienced declines (Woodhams et al. 2006b). When four species of Australian frogs were experimentally infected with *B. dendrobatidis*, those with more potent anti-*B. dendrobatidis* skin activity were more resistant (Woodhams et al. 2007).

### Role of adaptive immunity in resistance to *B. dendrobatidis*

There have been very few studies that examine the role of the adaptive (lymphocyte-mediated) components of the immune system on resistance to *B. dendrobatidis*. Using *Xenopus laevis* as a model species, we showed that following an experimental exposure to infectious *B. dendrobatidis* zoospores, intact frogs developed a mild infection over 20–30 days that declined by 45 days (Ramsey et al. 2010). Sublethal X-irradiation of frogs decreased leukocyte numbers in the spleen and resulted in greater susceptibility to infection and greater loss of weight (Ramsey et al. 2010). The X-irradiation did not damage granular glands or impair the ability of the irradiated frogs to secrete antimicrobial peptides. Immunization against *B. dendrobatidis* induced elevated pathogen-specific IgM and IgY serum antibodies. Mucus secretions from *X. laevis* previously exposed to *B. dendrobatidis* contained significant amounts of IgM, IgY, and IgX antibodies that were capable of binding to *B. dendrobatidis* (Ramsey et al. 2010). These data strongly suggest that adaptive immune defenses are involved in the resistance of *X. laevis* to otherwise lethal *B. dendrobatidis* infections. Although the data support a role for the adaptive immune system in resistance of some species to *B. dendrobatidis*, immunization with heat-killed *B. dendrobatidis* to induce a protective systemic immune response did not protect boreal toads

(*Bufo boreas*) or mountain yellow-legged frogs (*Rana muscosa*) when they were exposed to relatively high numbers of infectious zoospores (Rollins-Smith et al. 2009; Stice and Briggs 2010). This suggests that conventional immunization protocols that induce elevated serum antibodies would not necessarily be protective against this pathogen, which is confined to the skin. However, protocols that would induce elevated mucosal antibody responses to *B. dendrobatidis* might be more successful.

### *Batrachochytrium dendrobatidis* can inhibit immune responses

Although our studies of the immune responses of *X. laevis* against *B. dendrobatidis* strongly support a role for the adaptive immune system in protection against this pathogen, it is still somewhat of a puzzle why the immune response is not consistently able to control this pathogen in many amphibian species. We hypothesize that factors produced by the fungus can interfere with the development of a robust cell-mediated immune response. Our ongoing studies show that soluble factors released by *B. dendrobatidis* can inhibit in vitro proliferation of both T-lymphocytes and B-lymphocytes from two anuran species. The factors are released by either live or dead sporangia, and they can induce apoptosis of the lymphocyte populations (J. Ramsey et al., unpublished data).

### Evidence that skin bacteria may be involved in resistance to *B. dendrobatidis*

The skin of amphibians is inhabited by a rich array of skin microbes (Harris et al. 2006, 2009a; Lauer et al. 2007, 2008; Woodhams et al. 2007b; Walke et al. 2011). While some of the species are potential pathogens, most are probably benign and may be beneficial. Studies of bacteria isolated from two salamander species (*Plethodon cinereus* and *Hemidactylium scutatum*) showed that a number of species of skin bacteria can inhibit the growth of *B. dendrobatidis* and the ascomycete fungus, *Mariannaea* sp., pathogenic to salamander eggs (Harris et al. 2006; Lauer et al. 2007, 2008). By scanning electron microscopy, bacteria associated with the skin were observed to divide, indicating that the cutaneous environment is appropriate for reproduction as well as for survival (Lauer et al. 2007). Bacteria isolated from *P. cinereus* produced the antifungal metabolites 2,4-diacetylphloroglucinol (2,4-DAPG), indole-3-carboxaldehyde, and violacein (Brucker et al. 2008a, 2008b). Other studies showed that *Rana pipiens*, the mountain yellow-legged frog,

*Rana muscosa*, and the glass frog, *Hyalinobatrachium colymbiophyllum*, also harbor a number of skin bacteria that can inhibit growth of *B. dendrobatidis* (Woodhams et al. 2007b, 2007c; Walke et al. 2011). *Rana muscosa* populations have declined in a pattern that suggests *B. dendrobatidis* is the leading cause (Rachowicz et al. 2006; Briggs et al. 2010; Vredenburg et al. 2010). Populations of *R. muscosa* that persist in the presence of *B. dendrobatidis* (*B. dendrobatidis*-endemic site) had a higher proportion of individuals with *B. dendrobatidis*-inhibiting bacteria than did those that were declining (*B. dendrobatidis*-epidemic site) (Woodhams et al. 2007b; Lam et al. 2009). Furthermore, pretreatment of *R. muscosa* juveniles with *Janthinobacterium lividum* (which produces the antifungal metabolite violacein) protected them from lethal *B. dendrobatidis* infections that occurred in untreated but *B. dendrobatidis*-exposed frogs (Harris et al. 2009b).

### Immune system reorganization at metamorphosis may impact the immune defense against *B. dendrobatidis*

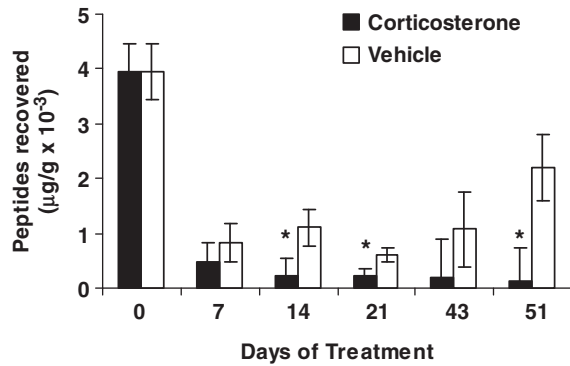
Previous studies of the ontogenetic development of the immune system of anuran amphibians showed that the immune system develops in two phases. The first is a rapid expansion during the tadpole's life followed by a significant loss of lymphocytes during the climax of metamorphosis (Du Pasquier and Weiss 1973; Rollins-Smith et al. 1984). This involution of the immune system at metamorphosis is followed by a second expansion of lymphocyte populations (Du Pasquier and Weiss 1973) and development of the adult-type recognition repertoire (reviewed by Rollins-Smith 1998). The loss of lymphocytes at metamorphosis appears to be due to the rapid increase in corticosteroid hormones that occurs during this period (Jaudet and Hatey 1984; Jolivet-Jaudet and Leloup-Hatey 1984; Rollins-Smith et al. 1997; Barker, et al. 1997; reviewed in Rollins-Smith 1998). The involution of the adaptive immune system at metamorphosis at the same time that the tadpole skin undergoes a transition to adult-type skin may provide a good opportunity for *B. dendrobatidis* to rapidly become established in the epidermis. Because the tadpoles' mouth parts harbor the fungus during larval life, emerging zoospores may infect the newly developing adult skin and establish overwhelming infections. In *B. dendrobatidis*-infected populations, die-offs of newly metamorphosing frogs have been reported in a series of studies (Berger et al. 1998; Bosch et al. 2001; Green et al. 2002; Rachowicz and Vredenburg 2004; Rachowicz

et al. 2006; Andre et al. 2008; Walker et al. 2010; Tobler and Schmidt 2010). Some antimicrobial peptides can be detected in the skin secretions of the tadpoles of some species (Wabnitz et al. 1998; D. Woodhams et al. unpublished data). However, other species do not appear to express defensive antimicrobial peptides until the granular glands mature at the time of metamorphosis (Bovbjerg 1963; Reilly et al. 1994; Clark et al. 1994). These newly formed reservoirs of antimicrobial peptides may begin to protect against *B. dendrobatidis* if the numbers of zoospores in the environment are limited.

### Stress that elevates corticosteroid hormones may interfere with immune mechanisms of resistance to *B. dendrobatidis*

Previous studies have shown that amphibian lymphocytes are inhibited by nanomolar concentrations of corticosterone (a glucocorticoid), aldosterone (a mineralocorticoid) (Rollins-Smith et al. 1997) and the synthetic steroid dexamethasone (Ruben et al. 1994). These effects are reversible *in vitro* and *in vivo* by the corticosteroid receptor antagonist, RU486 (Rollins-Smith et al. 1997). Tadpoles also elevate corticosterone in response to food deprivation (Crespi and Denver 2005). Other natural events in the life of a frog that elevate corticosteroid hormones are metamorphosis (Jaudet and Hatey 1984; Jolivet-Jaudet and Leloup-Hatey 1984) and the breeding activity of explosive-breeding species (Mendonca et al. 1985; Orchinik et al. 1988). Thus, events in the life of amphibians, such as metamorphosis, intensive breeding, or decreased food resources, could result in a natural elevation of corticosteroid hormones, immune suppression, and greater susceptibility to *B. dendrobatidis* infections. The impact of corticosteroid hormones is not limited to lymphocyte-mediated responses. We have also shown that pharmacological concentrations of corticosterone will completely inhibit renewal of amphibian skin peptides following norepinephrine-induced depletion of the peptides in adult *X. laevis* (Fig. 2).

The physiological responses of frogs suffering from advanced stages of chytridiomycosis may also alter corticosteroid hormone levels. Development of chytridiomycosis resulted in elevation of corticosterone and a decrease in lymphocyte numbers in the blood of *Litoria caerulea* in the weeks preceding death induced by *B. dendrobatidis* (J. Peterson and M. Mendonca, unpublished data). Another study of the development of chytridiomycosis in Panamanian golden frogs (*Atelopus zeteki*) showed elevated levels



**Fig. 2** Depletion and renewal of cutaneous peptides is inhibited by corticosterone in adult *Xenopus laevis*. Cutaneous peptides were depleted by injection of 80 nmol/g of norepinephrine and quantified by MicroBCA assay as described by Ramsey et al. (2010). Half of the peptide-depleted frogs were injected with corticosterone (25 µg/g) and the other half were injected with vehicle five times weekly.  $N=49$  frogs injected at Day 0 with norepinephrine and 4 or 5 frogs from each treatment group were injected again with 80 nmol/g norepinephrine at each of the subsequent time point. Peptides were significantly reduced from those of Day 0 inductions in vehicle-treated control frogs until Day 51 when they began to recover. Concentrations of peptides recovered from corticosterone-injected frogs were significantly less than those of Day 0 frogs at all time points and less than vehicle-injected controls at Days 14, 21 and 51 (\*) by a one-tailed Student's *t*-test,  $P \leq 0.05$ .

of cortisol in the feces of heavily infected frogs (M. Becker et al., unpublished data). Thus, the physiological response of *L. caerulea* and *A. zeteki* to *B. dendrobatidis* infection may impair immune defenses and exacerbate the outcome of the disease.

### Lower temperatures can affect immune responses to *B. dendrobatidis*

A number of previous studies have shown that the immune system of amphibians undergoes involution during natural hibernation (Cooper et al. 1992; Plytycz and Bigaj 1983; Plytycz et al. 1991; Miodonski et al. 1996; reviewed by Rollins-Smith and Woodhams 2011). Antibody responses and skin-graft rejection (a classical T-cell mediated response) are also significantly impaired by lower temperatures (Cohen 1966; Cone and Marchalonis 1972; Lin and Rowlands 1973; Jozkowicz and Plytycz 1998; reviewed by Rollins-Smith and Woodhams 2011). Chytridiomycosis die-offs have been associated with higher elevation and lower temperatures in a number of studies (Bradley et al. 2002, 2004; Drew et al. 2006; McDonald et al. 2005; Kriger et al. 2007; rev. in Fisher et al. 2009). Prevalence of infection can increase in cooler seasons (McDonald et al. 2005;

Woodhams and Alford 2005). Furthermore, *B. dendrobatidis* is able to persist at low temperatures (4–14°C). That is, more zoospores are shed and are active over a longer period of time (Woodhams et al. 2008). Whether or not chytridiomycosis (the disease) develops likely depends on multiple factors including the ability to produce defensive antimicrobial peptides in the skin. A recent analysis of the pattern of declines of members of the genus *Atelopus* in Central America (generally thought to be due to chytridiomycosis) attributes the pattern to extreme temperature variations that may have impaired immunity (Rohr and Raffel 2010). Because *B. dendrobatidis* thrives at low temperatures, the balance between immunity and disease may shift when temperatures fall.

### Environmental chemicals may affect immunity

Exposure to pesticides can alter immune defenses of amphibians and their susceptibility to parasites or pathogens (reviewed by Lannoo 2009; Rohr et al. 2009). For example, exposure to the chemicals atrazine, malathion, and esfenvalerate suppressed circulating eosinophils and led to increased infections by trematodes and increased limb malformations in *Rana sylvatica* (Kiesecker 2002). Taylor et al. (1999) showed that exposure to malathion increased the susceptibility of adult Woodhouse's toads (*Bufo woodhousii*) to the lethal effects of the bacterium *Aeromonas hydrophila*. A mixture of pesticides representative of wetland environments in southwestern Quebec suppressed some functions of the immune system of *X. laevis* and *R. pipiens* (Christin et al. 2003, 2004). There are other reports of long-term immunosuppression in *R. pipiens* exposed to environmental chemicals (Gilbertson et al. 2003; Gendron et al. 2003; Albert et al. 2007; Rohr et al. 2008; reviewed by Rohr and McCoy 2010). Skin-peptide defenses were significantly reduced after exposure to sublethal concentrations of carbaryl in foothill yellow-legged frogs (*Rana boylei*) or Southern bell frogs (*Litoria raniformis*) (Davidson et al. 2007; Schadich et al. 2009). The temporary change in availability of skin peptides did not result in greater susceptibility to chytridiomycosis (Davidson et al. 2007) or to the opportunistic bacterium *Klebsiella pneumoniae* (Schadich et al. 2009), but repeat exposures might impair this natural skin defense. Exposure of developing wood frogs (*R. sylvatica*) to glyphosate and *B. dendrobatidis* did not result in greater susceptibility to chytridiomycosis-induced mortality, and in this study, the pesticide treatment may have reduced



mortality due to *B. dendrobatidis* (Gahl et al. 2011). Thus, at the present time, there is no clear link between exposure to pesticides, immunosuppression, and increased susceptibility to *B. dendrobatidis*.

## Summary and future directions

In summary, current research supports the model of amphibian immune defenses against *B. dendrobatidis* or other pathogens shown in Fig. 1. Infectious zoospores or bacteria landing in the skin mucus must overcome chemical defenses, including antimicrobial peptides, lysozyme, secreted antibodies, and bacterial metabolites that may have antifungal or antibacterial activities. If *B. dendrobatidis* zoospores reach the growing epidermal cells, they would be expected to alter the properties of those cells and attract the attention of antigen-presenting cells such as dendritic cells or macrophages. These cells would be expected to recruit an adaptive cell-mediated and antibody-mediated lymphocyte response. However, factors produced by the fungus can interfere with lymphocyte responses by induction of apoptosis of lymphocytes.

The immune defense against chytridiomycosis is affected by a variety of factors that may alter the overall survival of the host. Because tadpoles carry mild infections in their mouthparts, changes in the natural immune system at metamorphosis may result in greater susceptibility to the development of the disease at that time. Stress due to a number of environmental factors including poor nutrition in marginal habitats and enhanced activity during breeding may elevate corticosteroids. If infection by *B. dendrobatidis* impairs ion transport in the skin, the endocrine response that balances cutaneous ion transport may also elevate glucocorticoid or mineralocorticoid responses resulting in immune suppression. The immune system of amphibians is highly temperature sensitive, and even small reductions in temperature may impair immunity. Environmental chemicals may also impair innate and adaptive immune responses. Although there has been great progress in understanding the immune defenses of amphibians against *B. dendrobatidis*, many questions remain unanswered. What are the mechanisms by which this pathogen prevents an effective immune response? Is it possible to develop an immunization strategy that will protect vulnerable species? Can skin bacteria provide an effective defensive barrier? How do environmental chemicals affect the skin's defenses against this pathogen? Because this pathogen has dramatically affected amphibian populations around the world,

continued research is needed to address these questions.

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